

APPLICATION FOR LETTERS PATENT

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Title: METHODS AND APPARATUS FOR IMPROVED
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TITLE OF THE INTENTION

**METHODS AND APPARATUS FOR IMPROVED
LUMINESCENCE ASSAYS USING MICROPARTICLES**

CROSS-REFERENCE TO RELATED APPLICATION

This application is a continuation-in-part of U.S. Application Serial No. 08/954,355 (Attorney Docket No. 370068-9045), filed October 20, 1997, incorporated herein by reference, which is a continuation of U.S. Application Serial No. 08/437,348 (Attorney Docket No. 370068-9040), filed May 9, 1995 (now U.S. Patent No. _____), incorporated herein by reference.

FIELD OF THE INVENTION

This application relates generally to methods and compositions for conducting binding assays, more particularly to those which measure the presence of an analyte of interest by measuring electrochemiluminescence emitted by one or more labeled components of the assay system. More specifically, the invention relates to precise, reproducible, accurate homogeneous or heterogeneous specific binding assays of improved sensitivity using electrochemiluminescent components.

BACKGROUND OF THE INVENTION

Numerous methods and systems have been developed for the detection and quantitation of analytes of interest in biochemical and biological substances. Methods and systems which

1 are capable of measuring trace amounts of microorganisms,
2 pharmaceuticals, hormones, viruses, antibodies, nucleic acids and
3 other proteins are of great value to researchers and clinicians.

4 A very substantial body of art has been developed based
5 upon binding reactions, e.g., antigen-antibody reactions, nucleic
6 acid hybridization techniques, and protein-ligand systems. The
7 high degree of specificity in many biochemical and biological
8 binding systems has led to many assay methods and systems of
9 value in research and diagnostics. Typically, the existence of
10 an analyte of interest is indicated by the presence or absence of
11 an observable "label" attached to one or more of the binding
12 materials.

13 Electrochemiluminescent (ECL) assays provide a
14 sensitive and precise measurement of the presence and
15 concentration of an analyte of interest. Such techniques use
16 labels or other reactants that can be induced to luminesce when
17 electrochemically oxidized or reduced in an appropriate chemical
18 environment. Such electrochemiluminescence is triggered by a
19 voltage imposed on a working electrode at a particular time and
20 in a particular manner. The light produced by the label is
21 measured and indicates the presence or quantity of the analyte.
22 For a fuller description of such ECL techniques, reference is
23 made to US Patent No. 5,221,605, US Patent No. 5,591,581, US
24 Patent No. 5,597,910, PCT published application W090/05296, PCT
25 published application W092/14139, PCT published application

1 WO90/05301; PCT published application WO96/24690, PCT published
2 application US95/03190, PCT application US97/16942, PCT published
3 application US96/06763, PCT published application W095/08644, PCT
4 published application WO96/06946, PCT published application
5 WO96/33411, PCT published application WO87/06706, PCT published
6 application WO96/39534, PCT published application WO96/41175, PCT
7 published application WO96/40978, PCT/US97/03653 and US patent
8 application 08/437,348 (U.S. Patent No. 5,679,519). The
9 disclosures of the aforesaid applications are incorporated by
10 reference. Reference is also made to a 1994 review of the
11 analytical applications of ECL by Knight, et al. (Analyst, 1994,
12 119: 879-890) and the references cited therein. The disclosure
13 of the aforesaid articles are also incorporated by reference.

14 While electrochemiluminescence assays are significantly
15 improved over chemiluminescence, fluorescence, ELISA and
16 radioisotope-based assays, as well as other assay techniques,
17 there is always a desire to improve assays by increasing the
18 signal or modulation signal obtained from a binding event. By
19 doing so one can improve the ratio of signal to background noise
20 and, therefore, the sensitivity of the assay. Increasing the
21 signal of an ECL assay also has several instrumental advantages
22 including the following: i) less sensitive (and less expensive)
23 light detection systems are required; ii) smaller samples are
24 required; iii) electrodes and instrumentation may be miniaturized

1 so as to allow for instruments that are smaller and/or devices
2 that run many assays concurrently in a small area.

3
4 OBJECTS OF THE INVENTION

5 1. It is therefore a primary object of this invention
6 to provide methods, reagents and compositions, for conducting of
7 electrochemiluminescence binding assays which improve one or more
8 characteristics of the assay or the instruments used to conduct
9 the assay.

10 2. It is a further and related object of this
11 invention to increase the number of photons emitted per binding
12 event in an electrochemiluminescence assay.

13 3. It is a further and related object of the
14 invention to improve the sensitivity of electrochemiluminescence
15 assay by increasing the signal and thereby increasing the ratio
16 of signal to background.

17 4. It is still a further and related object of the
18 invention to reduce the sensitivity requirements for the light
19 detection system used in electrochemiluminescence instruments.

20 5. It is still a further and related object of the
21 invention to miniaturize ECL electrodes and instrumentation and
22 thereby decrease the size the instruments themselves.

23 6. It is still a further and related object of the
24 invention to miniaturize ECL electrodes and instrumentation and

1 thereby increase the number of assays that can be run
2 concurrently in one device.

3
4 SUMMARY OF THE INVENTION

5 These and other objects of the invention are achieved
6 using microparticles comprised of an electrically conductive
7 material having (a) one or more copies of an assay ligand
8 immobilized on its outer surface, and (b) a plurality of
9 electrochemiluminescent moieties immobilized on its outer
10 surface. The assay ligand may be linked to the
11 electrochemiluminescent moiety. More specifically, it has now
12 been found that colloidal gold is a highly advantageous
13 conductive material with which to form microparticles. Colloidal
14 gold particles having one or more assay ligands immobilized on
15 its outer surface and a plurality of ECL moieties immobilized on
16 its outer surface can be used in a wide range of assay formats,
17 including those based on detecting the ECL from moieties
18 immobilized on the particle and those based on the modulation by
19 the particles of the ECL from free ECL moieties in solution. The
20 objects of the present invention may also be achieved using
21 microparticles that do not comprise electrically conductive
22 material.

23 Assays for an analyte of interest present in a sample
24 are conducted by (a) forming a composition comprising (i) the
25 sample, and one or more microparticles of the invention; (b)

1 incubating said composition to form a complex; (c) causing the
2 complex to bind to an assay-ligand immobilized on an electrode;
3 and (d) conducting an electrochemiluminescence measurement in the
4 presence of electrochemiluminescence reactants.

5 Assays employing bound ECL moieties can be carried out
6 in a similar sequence of steps. The complex formed includes an
7 assay ligand, the microparticle of the invention (wherein said
8 microparticle contains ECL moieties) and the assay-ligand
9 immobilized on the electrode so as to furnish a plurality of ECL
10 moieties at the electrode. The electrochemiluminescence
11 measurement is conducted there in the presence of reactants.
12

13 DETAILED DESCRIPTION OF THE INVENTION

14 Definition of Terms

15 "Assay-ligand" means a binding substance which may be
16 an analyte or an analog thereof; a binding partner of the analyte
17 or an analog thereof; additional binding partners of the binding
18 partner or analog thereof; or a reactive component capable of
19 reacting with the analyte, an analog thereof or a binding partner
20 or analog thereof. These species can be linked to a combination
21 of one or more binding partners and/or one or more reactive
22 components and/or an analyte or its analog or a combination
23 thereof. It is also within the scope of the invention for a
24 plurality of the aforementioned species to be bound directly, or
25 through other molecules to an analyte or its analog.

1 The term assay-ligand, therefore, includes analytes that can
2 be measured by a binding assay, e.g., proteins (including
3 oligopeptides, polypeptides, glycoproteins, lipoproteins and
4 peptide analogs), nucleic acids (including mononucleotides,
5 oligonucleotides, polynucleotides, ribonucleic acids,
6 deoxyribonucleic acids, and nucleic acid analogs), lipids,
7 steroids, carbohydrates (including sugars and polysaccharides),
8 porphyrins, alkaloids, nucleotides, nucleosides, amino acids,
9 fatty acids, viruses, microorganisms, and biological cells
10 (including prokaryotic and eukaryotic cells) and subcellular
11 particles derived therefrom. The analytes may be, for example,
12 antibodies, enzymes, receptors, ligands, hormones,
13 pharmacological agents, cellular metabolites, toxins, pathogens,
14 carcinogens, vitamins, transport proteins, structural proteins,
15 cofactors, nucleic acid binding molecules, and nucleic acid
16 sequences (including sequences characteristic of pathogens ,
17 disease states, or susceptibility to diseases). The "binding
18 partner" or "additional binding partners of the binding partner"
19 may be, for example, antibodies (e.g., antibodies that bind to
20 the analyte, to other antibodies, to nucleic acids, or to haptens
21 linked to an analyte or binding partner), haptens recognized by
22 an antibody, ligands recognized by receptors, receptors, nucleic
23 acids, nucleic acid binding compounds, avidin, streptavidin, or
24 biotin (or a biotin-labeled macromolecule).

1 The term "ECL moiety" and "TAG" are used
2 interchangeably to refer to labels or other reactants that can be
3 induced to luminesce (or can be chemically transformed into
4 species that luminesce) when electrochemically oxidized or
5 reduced in an appropriate chemical environment. It is within the
6 scope of the invention for the species termed "ECL moiety", and
7 "TAG" to be linked to an assay ligand. The term "TAG1" is used
8 to refer to ECL moieties comprising ruthenium tris(bipyridyl) and
9 derivatives thereof. It is within the scope of the invention for
10 a species termed "TAG1" to be linked to an assay ligand. The
11 terms "ECL coreactant" or "coreactant" are used to refer to
12 reagents or analytes that promote electrochemiluminescence from
13 an ECL moiety.

14 The terms detection and quantitation are referred to as
15 "measurement", it being understood that quantitation may require
16 preparation of reference compositions and calibrations.

17
18 **Brief Description Of The Drawings**

19 Fig. 1 is a schematic representation of a microparticle
20 of the invention having antibodies immobilized upon it, wherein
21 said antibodies are linked to ECL moieties, and a sandwich
22 immunoassay employing that microparticle.

23 Fig. 2 is a schematic representation of another
24 embodiment of the invention employing a microparticle of the
25 invention having antibody immobilized upon it and a sandwich

1 immunoassay using that particle in an electrochemiluminescence
2 assay using free ECL moieties in solution.

3 Fig. 3 is a plot of the ECL signal (background
4 corrected) measured in an ECL sandwich immunoassay for AFP as a
5 function of the concentration of AFP in the sample, wherein the
6 secondary antibody was labeled with TAG 1 and coated on the
7 surface of colloidal gold particles. The ECL signal obtained
8 when the TAG 1 labeled antibody was not linked to a microparticle
9 is also given for comparison.

10 Fig. 4 is the ECL signal (background corrected)
11 measured in an ECL sandwich immunoassay for AFP as a function of
12 the concentration of AFP in the sample, wherein the secondary
13 antibody was labeled with TAG1 and coated on the surface of
14 colloidal titanium oxide particles.

15 Fig. 5 is a comparison of the ECL signal and surface
16 fluorescence for TAG1 and biotin-labeled polylysines on a
17 streptavidin-coated electrode surface, wherein the data points
18 represent different concentrations of polymer or different ratios
19 of the number of TAG 1 more then per polymer strand.

20 21 The Microparticles of the Invention

22 It has been found that significant benefits are
23 achieved in electrochemiluminescence reactions using
24 microparticles comprised of an electrically conductive material
25 having (a) one or more copies of an assay-ligand immobilized on

1 its outer surface and (b) a plurality of electrochemiluminescent
2 moieties immobilized on its outer surface. The micro-particles
3 may have a coating thereupon upon which the assay-ligand and
4 electrochemiluminescent moieties are immobilized. In another
5 embodiment the conductive microparticles have a plurality of
6 copies of an assay-ligand labeled with an electrochemiluminescent
7 moiety immobilized on its outer surface.

8 The microparticles of the invention are preferably
9 comprised of a highly conductive and/or semiconductive material.
10 In an especially preferred embodiment of the invention, the
11 microparticles are colloidal gold particles. The preparation of
12 conductive particles is well known in the art (see, e.g.,
13 *Nanomaterials: Synthesis, Properties, and Applications*,
14 Edelstein, A.S. et al. Ed., Inst. Of Physics Publications:
15 Philadelphia, 1996 and Fendler et al., *Adv. Mater.*, 1995, 7:607).
16 For example, conductive microparticles may be prepared that
17 comprise metals, for example, gold, silver, platinum, palladium,
18 zinc, iron, nickel, lead, and copper (see, e.g., Bradley, J.S.,
19 *Clusters Colloids*, 1994, 459-544 and Frens, G., *Nature Physical*
20 *Science*, 1973, 241:20-22). The particles may comprise alloys of
21 more than one metallic element. Conductive microparticles may
22 comprise graphitic carbon (e.g., carbon black, graphitic
23 nanotubes, or fullerenes); see, e.g., *Handbook of Carbon*,
24 *Graphite, and Fullerenes*, Pierson, H.O., Ed., Noyes Publications,
25 1993; *Carbon Nanotubes*, Endo, M., Ed., Pergamon Pr.: Oxford,

1 1996; and US Patent No. 4,663,230. Conductive microparticles may
2 comprise organic conductors, for example, polypyrrole,
3 polythiophene, polyaniline, and polyacetylene (for a review of
4 organic conductors see, e.g., *Conjugated Conducting Polymers*, H.
5 Keiss, Ed., Springer-Verlag: New York, 1992; for a review of
6 bioanalytical applications of some organic conductors, see, e.g.,
7 Barisci et al., *Trends in Polym. Sci.*, 1996, 4:307-311; for a
8 description of colloids prepared from organic conductors, see,
9 e.g., US Patent No. 5,252,459 and *Colloidal Polymer Particles*,
10 Buscall, R., Ed., Academic Press: San Diego, 1995). The
11 microparticles of the invention may comprise a semi-conductor,
12 for example, a semi-conducting metal oxide. Some examples of
13 semiconducting metal oxides that have been incorporated into
14 microparticles include tin oxide (undoped or doped with antimony
15 or indium), titanium oxide, zinc oxide, and cuprous oxide. Other
16 semiconducting materials that have been incorporated into
17 microparticles (see, e.g., Murray et al., *J. Am. Chem. Soc.*,
18 1993, 115:8706-8715) include silicon, cadmium sulfide, cadmium
19 selenide, molybdenum sulfide, and zinc selenide. The
20 microparticles may be entirely composed of one or more conducting
21 and/or semiconducting materials or may also comprise an
22 insulating material. For example, conducting and/or
23 semiconducting particles have been formed by coating particles of
24 an insulating material (e.g., silica) with, e.g., organic

1 conductors, metals, or metal oxides (see, e.g., , US Patents No.s
2 H001447, 5512094, 5552086, and 5236737).

3 The microparticles of the invention may have a wide
4 variety of sizes and shapes. By way of example and not
5 limitation, microparticles may be between 5 nanometers and 10
6 micrometers. Preferably microparticles have sizes between 20 nm
7 and 200 nm. The particles may be spherical, oblong, rod-like,
8 etc., or they may be irregular in shape.

9 The microparticles of the invention preferably comprise
10 materials that are electrically conductive. In the context of
11 this application, conductive materials are materials that have
12 bulk resistivities of less than $1 \times 10^{13} \Omega\text{cm}$. We use the term
13 "very highly conductive" to refer to particles comprising
14 materials with bulk resistivities less than $1 \times 10^{-4} \Omega\text{cm}$. We use
15 the term "highly conductive" to refer to particles comprising
16 materials with bulk resistivities less than $1 \times 10^{-2} \Omega\text{cm}$. The
17 invention also includes the use of microparticles comprising
18 primarily non-conducting materials (e.g., silica, alumina,
19 polystyrene, polyacrylates, polyacrylamides, ceramics, glasses
20 and such). Microparticles of the invention may be solid or
21 porous. Microparticles of the invention may also include
22 macromolecules or aggregates thereof (e.g., a polymer, a
23 dendrite, a polysaccharide, a protein, nucleic acids, or other
24 biological macromolecules of appropriate size). Microparticles

1 may be inanimate or alternatively, may include animate biological
2 entities such as cells, viruses, bacterium and the like.

3 In one embodiment of the invention, the use of
4 microparticles as labels provides for improved signal in ECL
5 assays by providing a scaffold for multiple ECL moieties. While
6 the use of binding reagents comprising multiple ECL moieties is
7 known, the signal enhancement obtainable by that approach is
8 limited for the following reasons: i) the number of labels that
9 can be attached to a binding reagent is limited by the surface
10 area of the reagent; ii) multiple labeling of a binding reagent
11 may lead to denaturation and/or inactivation of the reagent; iii)
12 multiple labeling of a reagent may interfere with its ability to
13 bind other reagents, e.g., by blocking the active site; and iv)
14 multiple labeling of a binding reagent may lead to quenching of
15 the luminescent excited state of one or more labels due to
16 crowding of the labels on one, e.g., protein or nucleic acid. By
17 providing a scaffold for ECL moieties, said scaffold not being
18 involved in the binding event and said scaffold having a large
19 surface area compared to the binding reagent, microparticles
20 reduce or eliminate the limitations stated above.

21 In certain embodiments of the invention, the use of
22 microparticles as a scaffold for multiple ECL moieties provides
23 for reduced non-specific binding between the ECL labels and/or i)
24 other entities present in either the sample (e.g., proteins
25 nucleic acid and the like); ii) the assay reagents (e.g., assay

1 ligands); iii) the instrumentation/materials used to perform ECL
2 assays (e.g., a solid support, an electrode, a cell, and the
3 like). Reduced non-specific binding can advantageously improve
4 the performance of assay measurements in several ways, for
5 example: i) by decreasing background (non-specific) signals to
6 improve sensitivity and/or dynamic range; ii) by reducing or
7 eliminating the necessity for wash steps during the assay process
8 (thus reducing the cost, time, and complexity of ECL assays and
9 instrumentation); iii); by allowing a multiple of different assay
10 ligands to be present in the same assay (or reaction) media
11 without excessively interfering with each other or with other
12 assay reagents or ECL instrument, and iv) by allowing more ECL
13 moieties to be incorporated in an ECL label (without incurring
14 undue nonspecific binding), thus increasing the number of photons
15 emitted per binding event (which may improve sensitivity, dynamic
16 range, and/or reduce the cost of complexity of light detectors
17 used for ECL assays).

18 We have observed in many ECL assays using
19 microparticles that the nonspecific binding has been unexpectedly
20 low, even relative to the nonspecific binding observed for assay
21 ligands labeled with ECL moieties, wherein the ligands are free
22 in solution.

23 In certain embodiments, non-specific binding can be
24 further reduced by appropriate modification of the
25 microparticles. In one embodiment, the microparticle is coated

1 with a substance (e.g., an oligo- or poly-ethylene glycol moiety)
2 that resists adsorption of proteins (e.g., the coating of gold
3 particles with oligo-ethyleneglycol terminated alkane thiolates
4 is described by Weissbecker, et al., *Langmuir*, 1996, 12:3763-
5 3772). Alternatively, the microparticle can be coated with a
6 substance bearing hydrophilic or charged moieties. The coating
7 of colloidal particles with such substances is a well-known
8 method of preventing particles aggregation and is termed "steric
9 stabilization", see, e.g., Sato et al., *Stabilization of*
10 *Colloidal Dispersions by Polymer Adsorption*, Marcel Dekker, New
11 York, 1980. In another embodiment, the microparticle may be
12 coated with ECL moieties designed to reduce or resist non-
13 specific binding of biological molecules (e.g., proteins, nucleic
14 acid, or the like): such ECL moieties may incorporate for
15 example, oligoethylene glycol moieties, hydrophilic moieties,
16 and/or charged moieties. See, for example, published PCT
17 US97/04150 for a description of some ECL moieties designed to
18 reduce non-specific binding.

19 In a preferred embodiment of the invention, the
20 microparticles are comprised of a conductive material, preferably
21 a highly conductive material. The use of some conductive
22 particles as scaffolds for ECL moieties can lead to additional
23 enhancements in ECL when compared to certain non-conductive
24 particles. Without being bound by theory, it is believed that
25 this additional enhancement is due to the ability of the particle

1 itself to conduct electrons from the working electrode so as to
2 oxidize or reduce ECL moieties on its surface. In an especially
3 preferred embodiment of the invention, the particles are
4 comprised of a very highly conductive material.

5 In a preferred embodiment of the invention, the
6 microparticles comprise a material capable of acting as a working
7 electrode for inducing ECL from a certain ECL moiety and a
8 particular ECL coreactant (i.e., the material is "ECL active").
9 It is possible to determine if a material is ECL active for a
10 particular combination of ECL moiety and coreactant by testing
11 whether a sample of the material, when used as a working
12 electrode in an appropriate electrochemical cell under
13 appropriate conditions, induces ECL. Using this testing
14 procedure for selecting ECL-active and ECL-inactive materials
15 for the generation of ECL from ruthenium-*tris*-bipyridyl in the
16 presence of tripropylamine (TPA) we have found that gold,
17 palladium, platinum, indium and antimony doped tin oxide,
18 polythiophene, carbon electrodes and other materials are ECL-
19 active. Certain other materials, under certain conditions, are
20 ECL-inactive or only weakly ECL-active relative to other ECL
21 active materials. Materials may be active with respect to one or
22 more ECL labels and/or ECL coreactants and inactive with respect
23 to one or more different ECL labels and/or coreactants.

24 The microparticle of the invention is preferably
25 transparent to the wavelength of light emitted by the ECL label

1 so that the microparticle does not block emitted light from
2 reaching the light detector. Microparticles with this
3 advantageous property may be selected by matching the absorption
4 properties of the particles to the emission properties of the
5 label. Conductive microparticles that transmit in the visible
6 are known, e.g., tin oxide (preferably doped with indium or
7 antimony) transmits light of most visible wavelengths. Another
8 example, colloidal gold, absorbs green strongly but transmits
9 orange and red.

10 The microparticle of the invention is preferably
11 attached to an assay-ligand (e.g., a protein such as an antibody
12 or receptor, a nucleic acid probe, or a small molecule analyte of
13 interest such as a pharmacological agent or hormone). The
14 attachment of molecules (e.g., proteins, nucleic acids, and small
15 molecules) to microparticles is known in the art (see, for
16 example, the following texts, all of which are included by
17 reference: *Colloidal Gold: Principles, Methods and*
18 *Applications*, Vol. 1-3; Hayat, M.A. Ed., Academic Press: New
19 York, 1989; *Immobilization of Enzymes and Cells*, Bickerstaff,
20 G.F., Ed., Humana Press: Totowa, NJ, 1997; US Patent No.
21 5,252,459; and Mirkin et al., *Nature*, 1996, 382:607-609). The
22 assay-ligand may be immobilized by adsorption on the particle.
23 The adsorption of proteins and/or nucleic acids on
24 microparticles, e.g., gold, silver, silica, polystyrene and such,
25 is known. Nucleic acids and proteins can be adsorbed directly on

1 metals, such as gold and platinum, that have surfaces that behave
2 as soft acids (see, e.g., Flanagan et al., *Electron. Lett.*,
3 1984, 20:968-970). Proteins adsorb strongly to the surface of
4 hard acids, e.g., oxides such as silicon dioxide, tin oxide,
5 titanium oxide (see, e.g., Asanov et al., *J. Colloid Interface*
6 *Sci.*, 1997, 191:222-235). Proteins and nucleic acids may also be
7 adsorbed onto particles having hydrophobic or charged surfaces
8 (e.g., unmodified polystyrene particles or polystyrene particles
9 modified with charged moieties). Assay-ligands can be adsorbed
10 onto surfaces by modification of the assay-ligands with moieties
11 that are known to strongly adsorb on the surface, for example:
12 thiols will facilitate adsorption on gold, hydrophobic groups
13 will facilitate adsorption on hydrophobic surface (such as
14 polystyrene), and charged groups will facilitate adsorption on
15 surfaces of opposite charge). Assay-ligands may also be attached
16 covalently onto the microparticles, for example, by the coupling
17 of an assay-ligand comprising an activated silane to a silicon
18 oxide or metal oxide surface or by the coupling of an assay-
19 ligand to functional groups present on a polymeric particle.

20 Alternatively, the assay-ligand may be immobilized by
21 adsorption and/or covalent attachment to a "binding layer" coated
22 on the surface of the particle. For example, an assay-ligand may
23 be covalently attached to an oxide surface (e.g., silica or tin
24 oxide) by attachment to functional groups introduced on the
25 surface of the particle (these functional groups may be

1 introduced by methods well-known in the art, e.g., by coating the
2 particle with a self-assembled layer of a functionalized monomer
3 such as a silane. Similarly, an assay-ligand may be covalently
4 attached to the gold surface of a gold particle by coating the
5 particle by reaction with a functionalized thiol (e.g., to form a
6 self-assembled monolayer), see, e.g., US Patent No. 5,384,073.

7 Assay-ligands can be attached to microparticles by attachment to
8 an adsorbed layer of material (e.g., a protein or a polymer),
9 see, e.g., Mrsny et al., *Eur. J. Cell. Biol.*, 1988, 45:200.

10 Preferably, coatings for conductive particles may have one or
11 more of the following properties so as to promote the
12 microparticle to act as an electrode for generating ECL: i) the
13 binding layer is itself at least partially conductive; ii) the
14 binding layer is thin (preferably < 5nm) and/or iii) the binding
15 layer does not completely coat the surface of the microparticle
16 (e.g., there are defects in the binding layer so as for allow
17 conduction of electrons through or around the binding layer)
18 during the ECL reaction.

19 The electrochemiluminescent moiety can be immobilized
20 on the particle in several ways. The electrochemiluminescent
21 moieties may be directly attached to the immobilized assay-
22 ligand, i.e., the binding reagent. Alternatively, the ECL moiety
23 may be immobilized directly on the particle or a binding layer
24 thereon, e.g., to ensure that the ECL moiety does not interfere
25 with the activity of the assay-ligand. Such direct

1 immobilization could be by adsorption of the unmodified ECL
2 moiety (e.g., TAG1 adsorbs onto metals, hydrophobic surfaces, and
3 negatively charged surfaces) or by modification of the ECL moiety
4 with a group with a high affinity for the surface (e.g., a thiol
5 for gold). The ECL moiety may be covalently attached to the
6 surface, for example by the coupling of the ECL moiety comprising
7 an activated silane to a silicon oxide or metal oxide surface or
8 by coupling of ECL moiety to functional groups present on a
9 plastic particle. The ECL moiety may be immobilized by
10 attachment to a binding layer (e.g., an carboxylic acid or amine-
11 containing label can be attached to a protein-coated
12 microparticle by the formation of amide bonds). The ECL moiety
13 may also be incorporated in the particle, e.g., by blending it in
14 a plastic particle, by attaching it within the pores of a porous
15 particle, or by enclosing it in a liposome.

16 In some embodiments of the invention, the ECL
17 coreactant (or derivative thereof) is immobilized on the
18 microparticle (e.g., by adsorption or covalent attachment). In
19 said embodiments, the ECL moiety may be free in solution (i.e.,
20 the ECL coreactant acts as the detected label in an assay).
21 Alternatively, the ECL moiety and the ECL coreactant are both
22 immobilized on the microparticle so as to increase the efficiency
23 (through forcing the ECL coreactant and ECL moiety into
24 proximity) of reactions between the ECL moiety (or reaction

1 products thereof) and the ECL coreactant (or reaction products
2 thereof).

3 The invention is not limited in the ECL moieties and
4 ECL coreactants that may be used and can generally be applied to
5 any system of ECL moiety and/or coreactant. ECL moieties that
6 may be used include: transition metal complexes (e.g., of Ru, Os,
7 Cr, Cu, Ir, Pd, Pt, Re), polypyridyl complexes of transition
8 metals (especially, ruthenium, osmium and rhenium), lanthanide
9 chelates, luminol (and other chemiluminescent diacylhydrazides),
10 luciferase, acridinium esters, polyaromatic hydrocarbons (for
11 example, 9,10-diphenylanthracene-2-sulfonate), and such. ECL
12 coreactants may be species that undergo oxidation or reduction at
13 an electrode to give high energy intermediates. These
14 intermediates react in turn react with ECL moieties (or reaction
15 products thereof), the products of said reactions being capable
16 of emitting light. Examples of coreactants that may be used
17 include: amines, NADH, flavins, dansylated amines, oxalate,
18 persulfate, peracids, hydrogen peroxide, and such. Other ECL
19 moieties and coreactants that may be used include those disclosed
20 in the following publications (and references cited therein):
21 Bard, et al. (US Patent No. 5,238,808); Knight et al., 1994,
22 Analyst, 119:879-890; US Patent No. 5,591,581; US Patent No.
23 5,597,910; PCT published application WO90/05296; PCT published
24 application WO96/24690; PCT published application WO96/33411; PCT
25 published application WO96/39534, PCT published application

1 WO96/41175 and PCT published application WO96/40978; Watanabe,
2 et al., *Photochemistry and Photobiology*, 1992, 55:903-909; Knight
3 et al., *Analyst*, 1996, 121:101R-106R; Bruno et al., *J. Biolumin.*
4 *Chemilumin.*, 1996, 11:193-206; Irons et al., *Analyst*, 1995, 120:
5 477-483; Knight et al., *Analyst*, 1995, 120: 1077-1082.

6 In one embodiment of the invention, a plurality of ECL
7 moieties are linked to a polymer chain, wherein said polymer
8 chain is an assay ligand or, alternatively, wherein said polymer
9 chain is linked to an assay ligand. The polymer chain may be a
10 linear polymer or, alternatively, a branched polymer such as a
11 dendrimer. The polymer may contain monomer units capable of
12 being linked to an ECL moiety or an assay ligand (e.g., thiols,
13 aldehydes, carboxylic acids or activated derivatives thereof,
14 amines, disulfides, alcohols, and such). Alternatively, the ECL
15 moiety and/or assay ligand are linked to a monomer that is
16 incorporated into a polymer during the course of a polymerization
17 reaction.

18 The attachment of chemical groups (including biological
19 molecules) to polymers by the modification of preformed polymers,
20 and/or by the incorporation of modified monomers into a growing
21 polymer, is well known. The types of polymers that may be used
22 include, but are not limited to, polymers or copolymers
23 containing the following classes of monomeric units: vinyl units
24 (e.g., ethylene, propylene, acrylonitrile, acrylates,
25 acrylamides, styrene, vinylacetate, maleic anhydride), amino

1 and/or iii) one or more assay ligands. These species may be
2 located on the surface of the particle or within the interior.
3 For example, a microparticle may be formed by suspension
4 polymerization (e.g., of a crosslinked polymer). Microparticles
5 linked to ECL moieties, ECL coreactants, and/or assay ligands may
6 be formed by including in the polymerization reaction monomer
7 units linked to ECL moieties, ECL coreactants, and/or assay
8 ligands. Alternatively, one or more of these species may be
9 attached to the fully polymerized microparticles. In one
10 embodiment of the invention, the microparticle is porous so as to
11 allow the diffusion of reactive species into the particles (e.g.,
12 to allow the diffusion of ECL coreactants to ECL moieties within
13 the particle). In another embodiment of the reaction, the
14 microparticle is made of a conducting polymer (e.g., polypyrrole,
15 polyaniline, polythiophene, or polyacetylene) so as to ensure
16 efficient oxidation or reduction of electroactive species (e.g.,
17 ECL moieties and/or coreactants) throughout the particle.

18 19 Assays of the Invention Using Microparticles

20 Assays Using Microparticles Comprising ECL Moieties

21 The assays for an analyte-of-interest in a sample
22 comprise the steps of (a) forming a composition comprising (i) a
23 sample, (ii) a microparticle having one or more copies of a first
24 assay-ligand immobilized on its surface and a plurality of ECL
25 moieties immobilized on its surface (iii) a second assay-ligand

1 immobilized on an electrode; (b) incubating the composition to
2 form a complex; and (c) conducting an ECL measurement in the
3 presence of ECL reactants. Said first and second assay-ligands
4 may be the same or different. A complex is thus formed including
5 (i) a microparticle having one or more copies of an assay-ligand
6 immobilized on its surface, and a plurality of copies of an ECL
7 moiety immobilized on its surface, and (ii) an assay-ligand
8 immobilized on an electrode.

9 The formation of said composition comprising said
10 sample, said microparticle, and said second assay-ligand may be
11 one step or may be further subdivided into a plurality of steps.
12 For example, the sample and the microparticle may be combined and
13 incubated to form a complex that is then contacted with the
14 second assay-ligand immobilized on an electrode. Alternatively,
15 said sample and said second assay-ligand immobilized on an
16 electrode may be combined to form a complex on the electrode that
17 is then contacted with said microparticle.

18 In an alternate embodiment, the second assay-ligand is
19 immobilized on a solid-phase support other than an electrode,
20 said solid-phase support being capable of being collected at (or
21 brought into contact with) an electrode. Assays of this alternate
22 embodiment comprise the steps of (a) forming a composition
23 comprising (i) a sample, (ii) a microparticle having one or more
24 copies of a first assay-ligand immobilized on its surface and a
25 plurality of ECL moieties immobilized on its surface (iii) a

1 second assay-ligand immobilized on a solid phase support; (b)
2 collecting said solid-phase support at (or bringing said solid
3 phase support into contact with) an electrode; and (c) conducting
4 an ECL measurement in the presence of ECL reactants. Said first
5 and second assay-ligands may be the same or different. A complex
6 is thus formed including (i) a microparticle having one or more
7 copies of an assay-ligand immobilized on its surface, and a
8 plurality of copies of an ECL moiety immobilized on its surface,
9 and (ii) an assay-ligand immobilized on an solid-phase support.
10 Suitable apparatus and solid-phase supports (e.g., magnetic
11 beads) for carrying out assays according to this embodiment
12 include those disclosed in PCT published application WO92/14139
13 and PCT published application WO90/05301.

14 The methods and compositions of the invention may be
15 constructed in a wide variety of formats. Such formats include
16 formats known in the art such as sandwich assays and competitive
17 binding assays (see, e.g., the following references, hereby
18 incorporated by reference: *Nonradioactive Labeling and Detection*
19 *of Molecules*, Kessler, C., ed., Springer-Verlag: Berlin 1992; *The*
20 *Immunoassay Handbook*, Wild, D., ed., Stackton Press: New York
21 1994; and Keller, G.H.; Manak, M.M. *DNA Probes*, 2nd Ed.,
22 MacMillan Publishers Ltd.: London, 1993; *Tietz Textbook of*
23 *Clinical Chemistry 2nd Edition*, Burtis et al. Ed., W.B. Saunders
24 and Co.: Philadelphia, 1994). For example, a sandwich assay may
25 be performed so that a complex is formed including (i) a

1 microparticle having one or more copies of a first assay-ligand
2 immobilized on its surface, and a plurality of copies of an ECL
3 moiety immobilized on its surface, (ii) a second assay-ligand
4 immobilized on an electrode (and/or solid-phase support), and
5 (iii) an analyte, wherein said analyte is bound to both said
6 first assay-ligand and said second assay-ligand so as to link
7 said microparticle to said electrode (and/or solid-phase
8 support). In another example, a competitive binding assay may be
9 performed so that a complex is formed including (i) a
10 microparticle having one or more copies of a first assay-ligand
11 immobilized on its outer surface, and a plurality of copies of an
12 ECL moiety immobilized on its surface, and (ii) a second assay-
13 ligand immobilized on an electrode (and/or solid-phase support),
14 wherein said first assay-ligand is the analyte (or an analog
15 thereof), said second assay-ligand is a binding partner of said
16 analyte, and said first and second assay-ligand are bound to each
17 other so as to link said microparticle to said electrode (and/or
18 solid-phase support). Typically, in competitive binding assays,
19 the presence of the analyte of interest in a sample results in a
20 decrease in the number of said complexes and, therefore, in the
21 ECL signal. In an alternate embodiment of said competitive
22 binding assay, said second assay-ligand is the analyte (or an
23 analog thereof) and said first assay-ligand is a binding partner
24 of said analyte.

1 As described above, some assays of the invention use a
2 microparticle having one or more copies of an assay-ligand
3 immobilized on its surface and a plurality of ECL moieties
4 immobilized on its surface (i.e., the microparticle having a
5 plurality of ECL moieties immobilized on its surface is used as a
6 label for the assay-ligand). We have found that the use of said
7 particles gives an enhancement in the signal observed in an ECL
8 binding assay for an analyte-of-interest. This enhancement
9 occurs because microparticles can be used that have many more ECL
10 moieties than can typically be put on an assay-ligand by direct
11 attachment (and; therefore, more ECL moieties can be induced to
12 electrochemiluminescence per binding event of the assay). For
13 example, a labeled nucleic acid probe used in an ECL nucleic acid
14 hybridization assay typically has one ECL moiety per probe
15 molecule; a labeled antibody used in an ECL immunoassay will
16 typically have 1-10 ECL moieties per antibody. We have prepared
17 microparticles having more than 100 labels per particle. A rough
18 estimate of the numbers of ECL-moieties that can be put on a
19 solid particle can be determined from the ratio of the surface
20 area of the particle to the cross-sectional area of the labeling
21 reagent. The number of ECL moieties may be even higher if the
22 ECL moieties are incorporated within the volume of the
23 microparticle or a coating thereon.

**Assays Using Microparticles Comprised
of an Electrically Conductive
Material and Comprising ECL Moieties**

The assays for an analyte-of-interest in a sample comprise the steps of (a) forming a composition comprising (i) a sample, (ii) microparticles comprised of an electrically conductive material having one or more copies of a first assay-ligand immobilized on its surface and a plurality of ECL moieties immobilized on its surface (iii) a second assay-ligand immobilized on an electrode; (b) incubating the composition to form a complex; and (c) conducting an ECL measurement in the presence of ECL reactants. Said first and second assay-ligands may be the same or different in structure and/or specificity. A complex is thus formed including (i) microparticles comprised of an electrically conductive material having one or more copies of a first assay-ligand immobilized on its outer surface, and a plurality of copies of an ECL moiety immobilized on its surface, and (ii) a second assay-ligand immobilized on an electrode.

The formation of said composition comprising said sample, said microparticle comprised of an electrically conductive material, and said second assay-ligand may be one step or may be further subdivided into a plurality of steps. For example, the sample and the microparticle may be combined and incubated to form a complex that is then contacted with the second assay-ligand immobilized on an electrode. Alternatively, said sample and said second assay-ligand immobilized on an

1 electrode may be combined to form a complex on the electrode that
2 is then contacted with said microparticle.

3 In an alternate embodiment, the second assay-ligand is
4 immobilized on a solid-phase support other than an electrode,
5 said solid-phase support being capable of being collected at (or
6 being brought into contact with) an electrode. Assays of this
7 alternate embodiment comprise the steps of (a) forming a
8 composition comprising (i) a sample, (ii) microparticles
9 comprised of an electrically conductive material having one or
10 more copies of a first assay-ligand immobilized on its surface
11 and a plurality of ECL moieties immobilized on its surface (iii)
12 a second assay-ligand immobilized on a solid phase support; (b)
13 collecting said solid-phase support at (or bringing said solid-
14 phase support into contact with) an electrode; and
15 (c) conducting an ECL measurement in the presence of ECL
16 reactants. Suitable apparatus and solid-phase supports (e.g.,
17 magnetic beads) for carrying out assays according to this
18 embodiment include those disclosed in PCT published application
19 WO92/14139 and PCT published application WO90/05301.

20 The methods and compositions of the invention may be
21 constructed in a wide variety of formats. Such formats include
22 formats known in the art such as sandwich assays and competitive
23 binding assays (see, e.g., the following references, hereby
24 incorporated by reference: *Nonradioactive Labeling and Detection*
25 *of Molecules*, Kessler, C., ed., Springer-Verlag: Berlin 1992; *The*

1 *Immunoassay Handbook*, Wild, D., ed., Stackton Press: New York
2 1994; and Keller, G.H.; Manak, M.M. *DNA Probes*, 2nd Ed.,
3 MacMillan Publishers Ltd.: London, 1993; *Tietz Textbook of*
4 *Clinical Chemistry 2nd Edition*, Burtis et al. Ed., W.B. Saunders
5 and Co.: Philadelphia, 1994). For example, a sandwich assay for
6 an analyte of interest may be performed so that a complex is
7 formed including (i) a microparticle comprised of an electrically
8 conductive material and having one or more copies of a first
9 assay-ligand immobilized on its surface, and a plurality of
10 copies of an ECL moiety immobilized on its surface, (ii) a second
11 assay-ligand immobilized on an electrode (and/or solid-phase
12 support), and (iii) an analyte, wherein said analyte is bound to
13 both said first assay-ligand and said second assay-ligand so as
14 to link said microparticle to said electrode (and/or solid-phase
15 support). In another example, a competitive binding assay for an
16 analyte of interest may be performed so that a complex is formed
17 including (i) a microparticle comprised of an electrically
18 conductive material and having one or more copies of a first
19 assay-ligand immobilized on its outer surface, and a plurality of
20 copies of an ECL moiety immobilized on its surface, and (ii) a
21 second assay-ligand immobilized on an electrode (and/or solid-
22 phase support), wherein said first assay-ligand is the analyte
23 (or an analog thereof), said second assay-ligand is a binding
24 partner of said analyte, and said first and second assay-ligand
25 are bound to each other so as to link said microparticle to said

1 electrode (and/or solid-phase support). Typically, in competitive
2 binding assays, the presence of the analyte of interest in a
3 sample results in a decrease in the number of said complexes and,
4 therefore, in the ECL signal. In an alternate embodiment of said
5 competitive binding assay, said second assay-ligand is the
6 analyte (or an analog thereof) and said first assay-ligand is a
7 binding partner of said analyte.

8 As described above, some assays of the invention use a
9 microparticles comprised of a conductive material and having one
10 or more copies of an assay-ligand immobilized on its surface and
11 a plurality of ECL moieties immobilized on its surface (i.e., the
12 microparticles comprised of a conductive material and having a
13 plurality of ECL moieties immobilized on its surface is used as a
14 label for the assay-ligand). We have found that the use of said
15 particles gives an enhancement in the signal observed in an ECL
16 binding assay for an analyte-of-interest. This enhancement
17 occurs because microparticles can be used that have many more ECL
18 moieties than can typically be put on an assay-ligand by direct
19 attachment (and, therefore, more ECL moieties can be induced to
20 electrochemiluminescence per binding event of the assay). For
21 example, a labeled nucleic acid probe used in an ECL nucleic acid
22 hybridization assay typically has one ECL moiety per probe
23 molecule; a labeled antibody used in an ECL immunoassay will
24 typically have 1-10 ECL moieties per antibody. We have prepared
25 microparticles having more than 100 labels per particle. A rough

1 estimate of the numbers of ECL-moieties that can be put on a
2 solid particle can be determined from the ratio of the surface
3 area of the particle to the cross-sectional area of the labeling
4 reagent. The number of ECL moieties may be even higher if the
5 ECL moieties are incorporated within the volume of the
6 microparticle or a coating thereon.

7 The use of microparticles comprised of conductive
8 materials can lead to additional enhancements in ECL signal when
9 compared to microparticles comprised of non-conductive materials.
10 Without being bound by theory, it is believed that this
11 additional enhancement is due to the ability of the microparticle
12 itself to act as an electrode for oxidizing and/or reducing ECL
13 moieties on its surface. The size of a microparticle having a
14 plurality of ECL moieties immobilized thereon may prevent some of
15 said moieties from coming into close enough proximity to a
16 working electrode to be oxidized and/or reduced directly by the
17 electrode. It is believed that these proximity limitations are
18 overcome by the use of microparticles comprised of conductive
19 materials because the microparticle can carry electrical energy
20 from the working electrode to said ECL moieties and/or can act as
21 a working electrode to oxidize and/or reduce said ECL moieties
22 (as well as additional ECL moieties, reactants or cofactors in
23 solution).

24 Figure 1 is an illustration of one embodiment of the
25 invention. The figure illustrates a "sandwich" immunoassay using

1 a microparticle (101) comprised of a conductive material and
2 coated with a TAG-labeled antibody (102). The assay for an
3 analyte-of-interest (103) in a sample comprises the steps of (a)
4 forming a composition comprising (i) a sample including an
5 unknown concentration of the analyte-of-interest (103), (ii) a
6 microparticle (101) comprised of a conductive material and having
7 a plurality of TAG-labeled first antibodies immobilized on said
8 microparticle, wherein said first antibodies (102) are capable of
9 binding said analyte-of-interest (103), and (iii) a second
10 antibody (104) immobilized on a working electrode (105), wherein
11 said second antibody (104) is capable of binding said analyte-of-
12 interest (103); (b) incubating said composition to form a
13 complex; and (c) conducting an ECL measurement in the presence of
14 ECL reactants, wherein luminescence (106) is generated.

15
16 **Assays Using Microparticles Comprised**
17 **of an Electrically Conductive Material**
18

19 The assays for an analyte-of-interest in a sample
20 comprise the steps of (a) forming a composition comprising (i) a
21 sample, (ii) a microparticle comprised of an electrically
22 conductive material having one or more copies of a first assay-
23 ligand immobilized on its surface (iii) a second assay-ligand
24 immobilized on an electrode; (b) incubating the composition to
25 form a complex; and (c) conducting an ECL measurement in the
26 presence of ECL reactants. Said first and second assay-ligands

1 may be the same or different. A complex is thus formed including
2 (i) a microparticle having one or more copies of an assay-ligand
3 immobilized on its outer surface, and (ii) an assay-ligand
4 immobilized on an electrode.

5 The formation of said composition comprising said
6 sample, said microparticle comprising electrically conductive
7 material, and said second assay-ligand may be one step or may be
8 further subdivided into a plurality of steps. For example, the
9 sample and said microparticle may be combined and incubated to
10 form a complex that is then contacted with the second assay-
11 ligand immobilized on an electrode. Alternatively, said sample
12 and said second assay-ligand immobilized on an electrode may be
13 combined to form a complex on the electrode that is then
14 contacted with said microparticle.

15 In an alternate embodiment, the second assay-ligand is
16 immobilized on a solid-phase support other than an electrode,
17 said solid-phase support being capable of being collected at (or
18 brought into contact with) an electrode. Assays of this
19 alternate embodiment comprise the steps of (a) forming a
20 composition comprising (i) a sample, (ii) a microparticle having
21 one or more copies of a first assay-ligand immobilized on its
22 surface and a plurality of ECL moieties immobilized on its
23 surface (iii) a second assay-ligand immobilized on a solid phase
24 support; (b) collecting said solid-phase support at (or bringing
25 said solid phase support into contact with) an electrode; and (c)

1 conducting an ECL measurement in the presence of ECL reactants.
2 Suitable apparatus and solid-phase supports (e.g., magnetic
3 beads) for carrying out assays according to this embodiment
4 include those disclosed in PCT published application WO92/14139
5 and PCT published application WO90/05301.

6 The methods and compositions of the invention may be
7 constructed in a wide variety of formats. Such formats include
8 formats known in the art such as sandwich assays and competitive
9 binding assays (see, e.g., the following references, hereby
10 incorporated by reference: *Nonradioactive Labeling and Detection*
11 *of Molecules*, Kessler, C., ed., Springer-Verlag: Berlin 1992; *The*
12 *Immunoassay Handbook*, Wild, D., ed., Stackton Press: New York
13 1994; and Keller, G.H.; Manak, M.M. *DNA Probes*, 2nd Ed.,
14 MacMillan Publishers Ltd.: London, 1993; *Tietz Textbook of*
15 *Clinical Chemistry 2nd Edition*, Burtis et al. Ed., W.B. Saunders
16 and Co.: Philadelphia, 1994). For example, a sandwich assay may
17 be performed so that a complex is formed including (i) a
18 microparticle comprised of an electrically conductive material
19 and having one or more copies of a first assay-ligand immobilized
20 on its surface, (ii) a second assay-ligand immobilized on an
21 electrode (and/or solid-phase support), and (iii) an analyte,
22 wherein said analyte is bound to both said first assay-ligand and
23 said second assay-ligand so as to link said microparticle to said
24 electrode (and/or solid-phase support). In another example, a
25 competitive binding assay may be performed so that a complex is

1 formed including (i) a microparticle comprised of an electrically
2 conductive material and having one or more copies of a first
3 assay-ligand immobilized on its surface, and (ii) a second assay-
4 ligand immobilized on an electrode (and/or solid-phase support),
5 wherein said first assay-ligand is the analyte (or an analog
6 thereof), said second assay-ligand is a binding partner of said
7 analyte, and said first and second assay-ligand are bound to each
8 other so as to link said microparticle to said electrode (and/or
9 solid-phase support). Typically, in competitive binding assays,
10 the presence of the analyte of interest in a sample results in a
11 decrease in the number of said complexes and, therefore, in the
12 ECL signal. In an alternate embodiment of said competitive
13 binding assay, said second assay-ligand is the analyte (or an
14 analog thereof) and said first assay-ligand is a binding partner
15 of said analyte.

16 As described above, some assays of the invention use a
17 microparticle comprised of a conductive material and having one
18 or more copies of an assay-ligand). In assays using said
19 microparticles, the ECL signal may be generated from ECL moieties
20 in solution (as opposed to ECL moieties immobilized on said
21 microparticles), said microparticles acting to modulate the ECL
22 generated at a working electrode. Advantageously, the working
23 electrode is comprised of an electrode material that is inactive
24 or only weakly active for generating ECL from a particular ECL
25 moiety and/or ECL coreactant ("ECL-inactive"). Some examples of

1 electrode materials that may, under certain conditions, be ECL-
2 inactive for generating ECL from TAG1 in the presence of
3 tripropylamine include nickel and some compositions of stainless
4 steel. In addition, some metals (such as gold) and graphitic
5 materials become less ECL active (under certain conditions) after
6 electrochemical oxidation at high oxidation potentials.

7 Advantageously, the microparticle is comprised of an
8 ECL-active material. Some examples of materials that are ECL-
9 active include gold, platinum, carbon and tin oxide (said tin
10 oxide preferably doped with indium or antimony), and
11 polythiophene. Additional ECL-active and ECL-inactive systems
12 can be determined by testing electrodes for their ability to
13 generate ECL from specific systems of ECL labels and coreactants.
14 The formation of a complex including i) a microparticle comprised
15 of ECL-active material and having one or more copies of an assay-
16 ligand immobilized on its surface and (ii) an assay-ligand
17 immobilized on an electrode comprised of an ECL-inactive but
18 conducting material, will enable ECL to be induced from ECL
19 moieties in solution. The formation of said complex is,
20 therefore, detected as a modulation in the ability of the
21 electrode to induce ECL from ECL moieties in solution. The
22 advantage of this embodiment is that the ECL moieties are in
23 solution so the number of ECL moieties that can be induced to
24 emit ECL is not limited by the number of moieties that can be
25 immobilized in or on a particle of a given size. A high degree

1 of amplification can be achieved by using high concentrations of
2 ECL moieties in solution so that many ECL moieties are induced to
3 emit ECL.

4 Figure 2 is an illustration of one embodiment of the
5 invention. The figure illustrates a complex including i) a
6 microparticle (201) comprised of an ECL-active material and
7 having an antibody (202) immobilized thereon, wherein said first
8 antibody (202) is capable of binding an analyte-of-interest
9 (203); ii) an antibody (204) immobilized on an electrode (205)
10 comprised of an ECL-inactive material, wherein said antibody
11 (204) is capable of binding said analyte-of-interest (203) and
12 iii) said analyte-of-interest (203). The electrode itself (205)
13 is not capable of causing ECL from ECL moieties (206) in
14 solution, however, the ECL-active particle (201) is capable of
15 carrying electrical energy from the electrode and/or of acting as
16 an electrode so as to induce ECL (207) from ECL moieties (206) in
17 solution.

18 EXAMPLES

19 Instrumentation, Materials, and Methods

20 (1) Materials.

21 PBS-1 (100 mM sodium phosphate, 150 mM sodium chloride,
22 pH 7.8), Assay Buffer (ORIGEN Assay Buffer, a buffered solution
23 containing tripropylamine and detergent), and TAG1-NHS (a NHS
24 ester-containing derivative of ruthenium *tris*-bipyridyl) are all
25

1 products of IGEN International. Succinimidyl-4-(N-
2 maleimidomethyl)-cyclohexane-1-carboxylate (SMCC) was purchased
3 from Pierce Chemical Co. Antibody Diluent (Elecsys AFP Antibody
4 Diluent, Boehringer-Mannheim) is a mixture of blocking agents
5 suitable for conducting AFP assays. Calibration solutions
6 containing known amounts of AFP were prepared in Calibrator
7 Diluent (Elecsys AFP Calibrator Diluent, Boehringer-Mannheim), an
8 artificial serum substitute.

9 Sandwich immunoassays for AFP were conducted using an
10 antibody pair purchased from Genzyme (Cat. #MMA1010). The
11 capture antibody (Antibody #2) was labeled with biotin by
12 reacting the antibody (1 mg, 6.7 nmol, in 180 uL of 15 mM
13 phosphate, pH 7.4) with 40 ug (72 nmol) of biotin-LC-sulfo-NHS
14 ester (IGEN International). The biotin-labeled antibody was
15 purified by gel filtration and diluted in Antibody Diluent to a
16 concentration of 7.5 ug/mL. The detection antibody (Antibody #1)
17 was labeled with TAG1 by reacting the antibody (at a
18 concentration of > 1mg/mL in 15 mM phosphate, pH 7.4) with TAG1-
19 NHS (at a concentration of 10 mg/mL in 2.2 uL DMSO). The TAG1-
20 labeled antibody was purified by gel filtration using 5mM borate,
21 pH 8.8 as the eluent. The number of labels per protein was
22 determined by measuring the concentration of TAG1 moieties (by
23 optical absorption) and the concentration of protein (BCA Assay,
24 Pierce Chemical Co.).

Composite electrodes containing 27% (w/w) Hyperion Fibrils in co-ethylene-co-vinyl acetate (EVA) according to the procedure of PCT application US 97/16942. In brief, the materials were compounded and then extruded into sheets of flexible material that could be stamped or cut to form EVA-fibril composite electrodes. PCT application US 97/16942 also describes the procedure for coating the EVA-fibril composite with an immobilized layer of streptavidin. In brief, the procedure was as follows: i) oxidation of the EVA-fibril composite (for 20 min. in a mixture containing 20.7 g of chromium trioxide, 30 mL of water, and 10 mL of concentrated sulfuric acid); ii) treatment of the surface with 0.7 g of N-hydroxysuccinimide (NHS) and 1.6 g of 1-ethyl-3-dimethylaminopropyl-carbodiimide (EDC) in 50 mL of methylene chloride to form NHS esters and iii) treatment of the surface with a solution of streptavidin (5 mg in 50 mL of PBS-1) for 4 h to immobilize streptavidin on the composite. The chromic acid oxidation was sometimes replaced by treatment of the composite with an oxygen plasma (Advanced Plasma Systems Series C Reactor, 2000 W, 10 min., 300 mtorr) with similar results.

(2) ECL Measurements

ECL was measured in electrochemical cells designed to hold 3/16" or 5/16" discs of the composite material (working electrode). The electrode was sealed (with an o-ring) against an aperture to form one surface of the cell. The cell also included counter and reference electrodes. Light emitted from the working

1 electrode surface was measured with a photomultiplier tube (PMT).
2 The potential at the working electrode was controlled with a
3 potentiostat.

4 In a typical ECL experiment, the cell was filled with
5 Assay Buffer and the potential at the working electrode was
6 ramped from 0 V to -0.8V to 2.3 V at a scan rate of 0.1 V/s. The
7 peak ECL signal occurred typically at about 1.0 V. We report the
8 integrated photocurrent measured at the PMT in units of nA's. We
9 note that the signal is dependent on the gain of the PMT so data
10 shown for different experiments is not necessarily directly
11 comparable.

12 Binding reactions on the composite electrode surfaces
13 were sometimes carried out in the ECL cell. In other cases, we
14 carried out the binding reactions in a separate container then
15 transferred the composite electrodes to the ECL cell for the
16 measurement of ECL.

18 EXAMPLE I

19 **Synthesis Of Gold Colloids With A Diameter Of ~ 40 nm.**

20 The colloids were prepared by the procedure of G. Frens
21 (*Nature Phys. Sci.* 1973, 241, 20-22). A solution of HAuCl_4
22 (0.01% by weight, 200mL) was heated to boiling in a glass beaker
23 covered with a crystallization dish. A solution of sodium
24 citrate (1% by weight, 2.0 mL) was then added. The combined
25 solutions were allowed to boil for another 10 min. and then

1 allowed to cool to room temperature. The resulting colloidal
2 suspension had a dark red color. The suspension was stored at
3 4°C until used.

4
5 **EXAMPLE II**

6 **Procedure For Coating Gold Colloids With TAG1-labeled Antibody.**

7 The stock suspension of colloidal gold (10.9 mL) was
8 adjusted to ~pH 8.8 by the addition of 65 uL of 0.1 M potassium
9 carbonate. TAG1-labeled antibody (83.3 uL of a 1.2 mg/mL
10 solution in 5 mM sodium borate, pH 8.8) was added with mixing to
11 give a final concentration of protein of 10 ug/mL. The
12 adsorption was allowed to proceed for 45 min at room temp.
13 Polyethylene glycol (PEG) was added (1 mL of a 1% w/v solution)
14 as a stabilizer. Centrifugation (270 x g, 20 min) removed
15 aggregated particles. The colloidal particles were centrifuged
16 to a pellet (17,000 x g, 1h) then resuspended in 5 mM sodium
17 borate, 0.1% PEG, pH 8.8. This process was repeated two more
18 times to ensure removal of excess non-adsorbed protein. Prior to
19 its use in an AFP assay, the antibody-coated gold colloid was
20 first diluted in Antibody Diluent to a concentration of 1.2 ug of
21 gold-bound antibody per mL of solution.

22
23 **EXAMPLE III**

24 **Procedure for Coating Colloidal Particles of Silica or Titanium**
25 **Dioxide with TAG1-Labeled Antibody.**

1 Colloidal silica particles with an average diameter of
2 40nm (Aerosil OX50, Degussa Corp.) were coated with TAG1-labeled
3 anti-AFP antibody by the procedure given below. Colloidal
4 titanium dioxide (Titanium Dioxide P25, Degussa Corp.) was coated
5 by an analogous procedure. A suspension containing the silica at
6 a concentration of 1 mg/mL in 5 mM borate, pH 8.9 was sonified
7 (Sonifier 250, Branson Ultrasonics), for 20 min. The suspension
8 was further diluted in the borate buffer to give a concentration
9 of silica of 30 ug/mL. TAG1-labeled anti-AFP antibody was added
10 to 5 mL of the suspension to give a concentration of 30 ug/mL and
11 the suspension was incubated for 45 min. at room temperature.
12 Polyethylene glycol (PEG) was added (0.5 mL of a 1% w/v solution)
13 as a stabilizer. The colloidal particles were centrifuged to a
14 pellet (15,000 x g, 45 min.) then resuspended in 5mM sodium
15 borate, 0.1% PEG, pH 8.9. This process was repeated two more
16 times to ensure removal of excess non-adsorbed protein. Prior to
17 its use in AFP assay, the antibody-coated silica was first
18 diluted in Antibody Diluent to a coated silica was first diluted
19 in Antibody Diluent to a concentration of 1.2 ug of gold-bound
20 antibody per mL of solution.

22 EXAMPLE IV

23 Procedure for Immobilizing Antibody and TAG1 on BSA-Coated
24 Colloidal Gold.

1 The stock suspension of colloidal gold (11 mL) was
2 adjusted to ~pH 8.8 by the addition of 77 uL of 0.1 M potassium
3 carbonate. Bovine serum albumin (BSA) was added (1.22 mL of a 10
4 mg/mL solution in 5 mM sodium borate, pH 8.8) with mixing to give
5 a final concentration of protein of 10 ug/mL. The adsorption was
6 allowed to proceed for 2 hr at room temp. The colloidal
7 particles were washed three times by centrifugation and
8 resuspension in 5 mM sodium borate, 0.02% Triton X-100, pH 8.8,
9 the final resuspension being in 4 mL of PBS-Triton (15 mM sodium
10 phosphate, 150 mM sodium chloride, pH 7.2). An aliquot of the
11 suspension (200 uL) was then treated with TAG1 NHS (10 uL of a
12 320 uM solution in DMSO) and SMCC (10 uL of a 3200 uM solution in
13 DMSO) to couple, respectively, TAG1 and maleimide groups to the
14 BSA layer. The reaction were allowed to proceed for 2 hrs then
15 the particles were washed two times with PBS-Triton and
16 resuspended in 100 uL of PBS-Triton-EDTA (PBS-Triton containing
17 EDTA at a concentration of 1 mM).

18 The thiol-reactive maleimide groups were used to
19 immobilized anti-AFP antibodies on the surface. The antibody
20 (Boehringer-Mannheim) had been treated prior to the
21 immobilization with a 17 fold excess of Traut's Reagent to
22 introduce thiol groups and had been purified by gel filtration
23 chromatography. The thiolated antibody (100 uL of a 0.5 mg./mL
24 solution in PBS-Triton-EDTA) was combined with the colloidal
25 suspension and the reaction was allowed to proceed overnight.

1 Cysteine, BSA and glycerol were added (to give final
2 concentrations of 1 mM, 1 mg/mL, and 10%, respectively) and the
3 particles were washed three times by centrifugation and
4 resuspension in a solution containing 15 mM phosphate, 150 mM
5 sodium chloride, 1mg/mL BSA and 10 % (w/v) glycerol. After the
6 last wash, the particles were resuspended in 100 uL of the wash
7 buffer. The suspension was diluted 1:5 in Antibody Diluent
8 before use in an AFP assay.

9
10 EXAMPLE V

11 AFP Assay Using Colloidal Gold Coated with TAG1-labeled Anti-AFP
12 Antibody.

13 The assay procedure was as follows: The streptavidin-
14 coated electrodes (3/16" diameter discs) were treated with 100 uL
15 of biotin-labeled antibody. The binding of the capture antibody
16 was allowed to proceed for 1 h with gentle shaking, then the
17 electrodes were washed twice with PBS (150 uL). The electrodes
18 were then treated with a mixture containing 105 uL of the
19 suspension of antibody-coated gold colloid (prepared as described
20 in Example II, ~1.5 TAGs per protein) and 20 uL of a solution
21 containing a known quantity of AFP dissolved in Calibrator
22 Diluent. The assay was allowed to proceed for 1 h with gentle
23 shaking. The electrodes were then washed with PBS (2 x 150 uL)
24 and stored in 50 mM phosphate, pH 7.5 containing 1% BSA. The

1 electrodes were placed in the ECL cell and ECL was then measured
2 in the presence of ECL Assay Buffer (IGEN International, Inc.)

3 Figure 3 gives the ECL signal minus the ECL background
4 as a function of the concentration of AFP in the sample (the
5 background is the ECL signal when no AFP was present in the
6 sample). For comparison, data is shown for the analogous assay
7 using free TAG1-labeled anti-AFP antibody in solution (as opposed
8 to antibody adsorbed on a colloid). The total concentration of
9 antibody in the assay using colloid-adsorbed TAG1-labeled
10 antibody was the same as the assay using free TAG1-labeled
11 antibody. The results clearly show a dramatic increase in the
12 signal minus background for the lower concentrations of AFP. We
13 also note that the antibody-coated gold colloid exhibited low
14 levels of non-specific binding; the background signal observed
15 for the antibody-coated gold reagent was slightly lower than that
16 observed for the free TAG1-labeled antibody in solution.

17 18 EXAMPLE VI

19 AFP Assay Using Colloidal Silica Coated with TAG1-Labeled Anti- 20 AFP Antibody.

21 The assay procedure was as follows: The streptavidin-
22 coated electrodes (3/16" or 5/15" diameter discs) were treated
23 with 100 uL of biotin-labeled antibody. The binding of the
24 capture antibody was allowed to proceed for 1 h with gentle
25 shaking, then the electrodes were washed twice with PBS (150 uL).

1 The electrodes were then treated with a mixture containing 100 uL
2 of the suspension of antibody-coated silica colloid (prepared as
3 described in Example III, ~2.7 TAGs per protein) and 20 uL of a
4 solution containing a known quantity of AFP dissolved in
5 Calibrator Diluent. The assay was allowed to proceed for 1 h
6 with gentle shaking. The electrodes were then washed with PBS (2
7 x 150 uL) and stored in 50 mM phosphate, pH 7.5 containing 1%
8 BSA. The electrodes were placed in the ECL cell and ECL was then
9 measured in the presence of Assay Buffer (IGEN International,
10 Inc.).

11 The table below gives the ECL signal measured for a
12 sample (containing AFP at a concentration of 5.6 IU/mL) minus the
13 ECL background as a function of the concentration of AFP in the
14 sample (the background is the ECL signal when no AFP was present
15 in the sample). For comparison, data is shown for the analogous
16 assay using free TAG1-labeled anti-AFP antibody in solution (as
17 opposed to antibody adsorbed on a colloid) or TAG1-labeled anti-
18 AFP adsorbed on colloidal gold particles. The total
19 concentration of antibody was the same in each case. The results
20 show that the ECL signal measured using the colloidal silica
21 reagent was higher than that observed for the free antibody in
22 solution but lower than that observed when using the colloidal
23 gold reagent. The background signals due to the three reagents
24 were roughly comparable.

Labeled Reagent	Signal - Background
Free TAG1-Ab	2
TAG1-Ab on Gold	91
TAG1-Ab on Silica	8

EXAMPLE VII

AFP Assay Using Colloidal Titanium Dioxide Coated with TAG1-Labeled Anti-AFP Antibody.

The assay procedure was as follows: The streptavidin-coated electrodes (3/16" or 5/15" diameter discs) were treated with 100 uL of biotin-labeled antibody. The binding of the capture antibody was allowed to proceed for 1 h with gentle shaking, then the electrodes were washed twice with PBS (150 uL). The electrodes were then treated with a mixture containing 100 uL of the suspension of the antibody-coated titanium dioxide colloid (prepared as described in Example III, ~ 1.5 TAGs per protein) and 20 uL of a solution containing a known quantity of AFP dissolved in Calibrator Diluent. The assay was allowed to proceed for 1 h with gentle shaking. The electrodes were then washed with PBS (2 x 150 uL) and stored in 50 mM phosphate, pH 7.5 containing 1% BSA. The electrodes were placed in the ECL cell and ECL was then measured in the presence of ECL Assay Buffer (IGEN International, Inc.). Figure 4 gives the ECL signal minus the ECL background as a function of the concentration of

1 AFP in the sample (the background is the ECL signal when no AFP
2 was present).

3
4 EXAMPLE VIII

5 AFP Assay Using Antibody and TAG1 Immobilized on BSA-Coated Gold
6 Colloids.

7 The assay procedure was follows: The streptavidin-
8 coated electrodes (3/16" or 5/15" diameter discs) were treated
9 with 100 uL of biotin-labeled antibody. The binding of the
10 capture antibody was allowed to proceed for 1 h with gentle
11 shaking, then the electrodes were washed twice with PBS (150 uL).
12 The electrodes were then treated with a mixture containing 100 uL
13 of the colloidal reagent (prepared as described in Example IV)
14 and 20 uL of a solution containing a known quantity of AFP
15 dissolved in Calibrator Diluent. The assay was allowed to
16 proceed for 1 h with gentle shaking. The electrodes were then
17 washed with PBS (2 x 150 uL) and stored in 50 mM phosphate, pH
18 7.5 containing 1% BSA. The electrodes were placed in the ECL
19 cell and ECL was then measured in the presence of Assay Buffer
20 (IGEN International, Inc.). The signal obtained for a sample
21 containing 56 IU/mL AFP was 472 nA.s compared to a background
22 signal of 121 nA.s.

EXAMPLE IX

The Use of Polymers Linked to a Plurality of ECL Moieties as
Labels for ECL Assays.

Polylysine (Sigma Chemical, P7890, Average Mol. Wt. 19kD) was labeled with biotin and TAG1 by the following procedure. Polylysine (11.3 mg) was dissolved in 6.93 mL of PBS-1 and the solution was filtered through a 0.22 um pore size filter. The solution was made basic with triethylamine (38 uL). The polylysine was labeled with TAG1 and biotin by reaction of the lysine amino groups with TAG1-NHS and biotin-LC sulfoNHS (IGEN International), respectively. Aliquots of the polylysine solution (800 uL, 70 nmol of polylysine) was treated with 5 equivalents (per polymer molecule) of biotin-LC sulfoNHS and between 1-100 equivalents (per polymer molecule) of TAG-NHS (by the addition of solutions prepared in DMSO) so as to give polymers comprising a constant small number of biotin moieties and varying numbers of TAG1 groups. After allowing 30 min. for the reactions to proceed to completion, the remaining unreacted amino groups were capped by the addition of an excess of succinic anhydride in DMSO. The polymers were purified by gel filtration chromatography on NAP-5 columns (Pharmacia) using PBS-1 as the eluent. A comparison of the solution fluorescence of polymer-bound TAG1 groups vs. non-polymer bound TAG1 showed similar emission yields indicating that little or no quenching occurs due

1 to the attachment of large numbers of TAG1 moieties on one
2 polymer chain.

3 Several polymers varying in the number of TAG1 moieties
4 per polymer were allowed to bind to streptavidin-coated chromic
5 acid-treated fibril-EVA composite electrodes. The concentrations
6 of the polymers were also varied so that electrodes were produced
7 having varying amounts of each polymer on the surface. After
8 binding, the electrodes were washed with PBS-1 and water to
9 remove excess non-bound polymer. The surface fluorescence due to
10 bound TAG1 moieties was measured at glancing angle using a Photon
11 Technology International Fluorimeter equipped with a solid sample
12 holder (the excitation wavelength was held at 450 nm, the
13 fluorescence signal was determined by integrating the signal
14 obtained by scanning the emission wavelength from 590-620 nm).
15 To measure ECL, the electrodes were placed in an ECL cell and ECL
16 was measured in the presence of ECL Assay Buffer. A log-log plot
17 of fluorescence vs. ECL for the various samples (Figure 5) shows
18 an approximately linear relationship between fluorescence and ECL
19 (a line with a slope of one is provided for comparison)
20 indicating that ECL can be efficiently induced from polymers
21 linked to multiple ECL labels. The fluorescence and ECL obtained
22 using a biotin- and TAG1-labeled IgG molecule is shown for
23 comparison.